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13. ABSTRACT *(Maximum 200 words)*

We have made substantial progress in the characterization of a new pre-clinical model system for the growth of patients' breast carcinoma which is now being used by our group for the study of breast tumor-specific immunity. During the past year, we have successfully grown over 40 different patients' breast carcinomas in a SCID mouse system. These tumors have grown with remarkably different histologies and growth rates; these differences are probably a reflection of the biological properties of the original tumors. Importantly, we have observed metastatic spread of several of these tumors within the SCID mouse making this model highly relevant to the most critical issues in the study of human breast cancer. We are characterizing these tumors for HER-2/neu expression and for DNA content using flow cytometry and are comparing this data to the same analysis conducted on the original patient's tumor. We have also observed the presence within the tumor-bearing SCID mouse of human tumor infiltrating lymphocytes that have apparently co-engrafted the SCID mouse along with the tumor. We are characterizing these lymphocytes for their surface phenotype and have noticed several mice in which there is a great expansion of these TIL. Most often, these cells are CD8 positive T cells, but we have also observed a large number of plasma cells and CD4 positive T cells. These data indicate that this new mouse model system for the growth of primary human breast carcinoma is a viable one with which preclinical studies directed toward immunotherapy can finally be conducted. During the upcoming year of this proposal, we will develop and test new strategies to enhance the anti-tumor activity of the human tumor-infiltrating lymphocytes we have observed. An abstract, manuscript and additional unexpected findings have been generated as a result of work during the first year.

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Elizabeth Repasky, Oct 29, 1995

PI - Signature Date

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(5) Introduction:

Pre-clinical research on the treatment of human breast cancer has been hampered by the lack of an appropriate experimental animal model in which the growth of primary breast carcinoma biopsies can be studied (1-7). Compounding this situation, It is very difficult to obtain enough primary breast tumor cells for most kinds of studies, including antigen identification or RNA and DNA isolation.

Most studies on human breast cancer rely on a few established immortal human breast cancer cell lines. Among the few cell lines which have been established in long-term culture, and which do grow in immunodeficient mice, most have been obtained from malignant pleural effusions (although see 9,10). Although majority of studies on human breast cancer are conducted on these cell lines, they may bear little resemblance to earlier malignancies found in primary breast carcinoma itself (1). An animal model that sustains the growth of patient breast tumors could be used not only to study the cellular and molecular properties of various breast carcinoma specimens and but also could be used to evaluate the effect of various immuno- or chemo-therapeutic agents or of various factors (such as diet) on breast tumor growth. Moreover, there is a great need for earlier detection markers and better prognostic indicators in the clinical evaluation of human breast carcinoma and knowledge of biological differences among various primary breast cancers would help to identify such factors and help in the choice of the best treatment modality.

Despite a longstanding effort however, human breast carcinomas have proven uniquely resistant to growth in a variety of animal model systems including the anterior chamber of the eye of guinea-pigs, lethally irradiated or thymectomized mice and nude and SCID mice (1-5) even when the animals are supplemented with estrogen. Apparently, human primary breast carcinoma cells require a very special micro environment for growth that has not yet been adequately replicated in animal models or *in vitro*.

At the time our original Army grant was submitted, we had been testing various protocols for improving the chances of obtaining growth of human breast tumors by using SCID mice; our initial and extensive attempts to engraft human breast tumors in SCID mice by inoculating tumor biopsy tissue or dispersed tumor cells subcutaneously or intra peritoneally were largely unsuccessful, confirming an earlier study by Phillips (5). However, we then tested a new idea and had just acquired our first preliminary data (on seven patients' tumors) when the grant was submitted and eventually funded.

Based upon our previous experience with the study of lung carcinoma in SCID mice, we based our grant application on the fact that this model (if it could be generalized to a larger number of patients) could be used to 1) study the patient's own immune response to her tumor and 2) identify strategies ways to enhance this response in a pre-clinical setting *if the SCID mouse could be found to harbor and support the activity of the patient's immune cells.* In this first year, we have made substantial progress toward achieving these aims, and these results are described in detail below. An Abstract presenting this new data was presented in the spring at the meeting of the American Association of Cancer Research in Toronto (attached to this report), and a full manuscript is very nearly complete; it will be sent to the U.S. Army within the next

two weeks.

(Note regarding Funding and Statement of Work)

It is probably important to note at this point that just before our grant award was to begin last Fall, the PI was notified of a large funding cut which was to be made. This was not expected based upon the critique we received which summarized the reviewers evaluation of not only the science in this proposal, but also its budget. No explanation for this cut could be obtained. In any case, the *entire* fourth year had to be deleted, as well as most of the equipment budget, and a third of the supply budget. Thus there were disappointing and unexpected changes which had to be made in the plans originally made in the Statement of Work. Nevertheless, in our first year, we have been very successful in generating a large amount of new and important information toward validating not only our new model, but in validating the opinions made in the scientific critique of our original proposal and we are sincerely hoping that a re-evaluation of the funding cut can somehow be made.

6) Body of Work

To begin this narrative, a summary of the Technical Objectives of the original grant is presented here:

1) To characterize the SCID mouse as a model for the growth of human breast cancer and autologous immunocompetent cells and correlate tumor growth parameters with patient's prognosis and expression of predictive markers of tumor malignancy.

2) To demonstrate and monitor specific anti-tumor reactivity of the human immunocompetent cells that are co-engrafted with autologous human breast tissue, and correlate these findings to the patient's prognosis and expression of predictive markers of malignancy.

3) To enhance the anti-tumor immunity of human immunocompetent cells in SCID mice by (1) in vivo or in vitro transfection of human breast tumor cells with genes encoding immunostimulatory proteins and (2) in vitro priming of immunocompetent cells with tumor antigens.

As would be expected from the Statement of Work, (1-36 months) most of our effort this past year was devoted to characterizing the growth of patients' primary breast tumors in the SCID mouse and also in determining if autologous lymphocytes could be found. As will be seen, we were very successful in achieving these goals and more during the past year.

a) A large percentage of patients' primary and recurrent breast tumors will grow in the SCID mouse.

We found that implantation of pieces of breast tumor biopsy material within the confines of the large gonadal fat pad (GFP) of female SCID mice resulted in sustained tumor growth. During the past year, 41 out of 46 surgical specimens implanted in a total 200 mice were

established as xenografts in SCID mice using the GFP protocol (Table 1). Much of this data is close to be being submitted for publication and the manuscript, along with color figures of the histology, will be submitted upon its completion to the U.S. Army Medical Research and Materiel Command. We are working very closely with the surgeons here at Roswell Park, Dr. Steve Edge and Mark Arredondo, to correlate as many clinical markers as possible with these new data. For example, as some tumors grow very quickly, whereas others do not, and since some exhibit metastasis within the SCID mouse while others do not, (see below) we are comparing these variables to 1) diagnosis and 2) disease progression in the patient.

b) The growth rates and histopathology of the tumors vary considerably

The initial growth rate of the various tumor biopsies that we received for this study are quite variable and at 3 months, tumors can range from 0.2-2.0 cm. in diameter. In many cases, the tumor biopsy remained a tiny size for about a year, and in many other cases, the tumor remained small for several months before starting to grow quite rapidly (reaching 1-2 cm diameter within 1 to 2 months) while still others grew rapidly right from the start. Tumors that grew the most rapidly are indicated on Table 1. In 5 cases, tumors which were originally established within GFP could later be passaged and grown in subcutaneous sites (see Table 1). Moreover, we have successfully passaged most of the tumors with a fast growth rate at least 2 times and several as many as 5 or 6 times in the mice and have also demonstrated that passaged tumor tissues which were recovered from liquid nitrogen storage can also grow within the GFP of SCID mice.

We have conducted a histological evaluation of many of the primary and passaged tumors listed in Table 1. In addition to the considerable variation in the rate of growth exhibited by various tumor xenografts, we have also noted significant variation in the histology of various tumors. In figures to be included with the manuscript, several representative photomicrographs of human breast tumor growth in SCID mice are presented.. The original pathology slides of several cancers of the invasive ductal type show tumor cells distributed in various configurations within the stromal regions of the gland. In the SCID mouse, both in the initial period of growth and in subsequent passages, these tumors often grow as a continuous, advancing sheet of uniformly-sized cells that insinuate themselves between adipocytes of the fat pad at the edges of tumor growth. In this type of growth pattern, we have not noticed a capsule or connective tissue sheath between the tumor and fat; instead, there appears to be a very close association between the adipocytes and the tumor cells. However, in some cases of IDC, we noted extensive cell size heterogeneity seen among the tumor cells with larger and smaller cells growing in close proximity and in still other cases of IDC and invasive lobular carcinoma, we noticed a "comedo-type growth pattern, similar to the pattern seen in the original pathology. A case of intraductal carcinoma also grew as distinct layers of cells that often surrounded fluid-filled or necrotic regions. In a case of papillary cystic cancer, tumor cells grew as distinct cords and spheres of cells within the fat tissue; these cords of cells were similar to regions seen in the original tumor biopsy. In examples of more slow growing tumors, as with the cases of IDC described above, actively-mitotic tumor growth was observed for long periods of time in the original xenograft and in subsequent passages.

In addition, we observed growth of both estrogen-receptor (ER) positive or negative

tumors. In separate experiments during passaging of ER-positive tumors (4 tumors), we observed that these tumors would not grow and eventually die if estrogen supplements were not given at the time of surgery. Estrogen receptor-negative tumors (4 tumors) however, grew well in the absence of estrogen (data not shown).

c) Metastatic growth occurs in several of the carcinomas implanted within the SCID mouse.

We have also examined the SCID mouse for evidence of metastasis in our human-SCID model and did observe gross metastasis in most of the fast growing tumors. The occurrence of gross tumors have been found in liver, lung, diaphragm, peyer's patch lymph nodes, and the abdominal wall and skin. Our histological examination of these tumors indicates that the histological pattern of the metastatic growth is similar or identical to the tumor growth found in the GFP where the passaged tumors were originally implanted. This is an extremely important finding and indicates that this model can be used to address the most critical issues of breast cancer treatment, including the study of metastasis. It will be extremely important to compare the cellular properties of the metastatic cells with those which remain at the site of implantation.

d) Finding of human lymphocytes which are present in the breast tumor-engrafted SCID mice (see 1995 AACR abstract by Xu et al., in Appendix)

As we had hoped based upon our previous lung tumor work, there are often substantial numbers of human lymphocytes which apparently engraft the SCID mouse from the original tumor biopsy (i.e., they were originally present as TIL cells). On several occasions, we have noted large aggregates of lymphocytes when the tumor failed to grow. We are analyzing these cells using immunophenotyping antibodies (including CD45, CD4 and CD8). Thus far, our analysis reveals that most cells are CD8 positive T lymphocytes, although there are also large numbers of plasma cells and CD4 positive lymphocytes. This observation is very important for the overall goal of this proposal because it provides the rationale for now pursuing the questions defined in Aim 3, .i.e, can we enhance the anti-tumor activity of these cells? We have just begun studies to characterize the serum of these SCID mice for the presence and anti-tumor reactivity of human antibodies. From our large panel of human tumors established during this past year, we have frozen away large quantities of tumor to be used as antigen in western analysis and in in vitro stimulation studies.

To prepare for the work to be conducted in Aim 3 during the next two years on enhancing anti-tumor responses, we have established a collaboration with Dr. Dr. Estuardo Aguilar-Cordova of Baylor College of Medicine to study the effects of gene transfection with various expression vectors producing heat shock proteins, and with Dr. Kenneth Grabstein (Corixa Corporation, Seattle, Wash) to examine the effects of in vitro immune stimulation of patients' lymphocytes.

e) Immunophenotyping of the breast tumor cells by flow cytometry

We are currently characterizing all of the tumors we have grown for HER-2/neu expression

and for DNA content and are comparing this data in the same tumors at different passages and with the original biopsy material. This work is being conducted in close collaboration with Dr. Carleton Stewart, Director of RPCI's Flow Cytometry Facility. Our initial data indicates a remarkable fidelity in both of these markers, and even after adriamycin treatment of the breast tumors (in the SCID mice) (See data attached to the Appendix). This evidence again supports the use of this model for pre-clinical studies as opposed to the use of cell lines which may not reflect very many of the biological properties of primary breast carcinomas.

7) Conclusions

During this past year, we have acquired substantial data which validate our new model, and which indicate that it will be useful in pre-clinical studies to evaluate strategies to enhance anti-tumor immunity. We are now beginning to more extensively characterize the autologous lymphocytes which we have discovered to co-engraft the SCID mouse along with the tumor biopsy, and which can sometimes expand to very large numbers. *We now have many different patients' tumors whose antigens we can assess by western blot analysis and through in vitro stimulation of patients peripheral blood lymphocytes and lymph node cells, and we have established excellent collaborations both within RPCI and at other centers to help us with this work.* We expect to move forward very rapidly during this second year and will be involved almost entirely in characterizing the patients' lymphocytes isolated from either peripheral blood or from tumor-draining lymph nodes in terms of their response to autologous tumor antigens, which we can now identify through our ability to grow up larger amounts of tumor cells.

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Table 1: Pathological Diagnosis and Tumor Growth in SCID Mice

Tumor #	Pathological Diagnosis	Nodal Involve-ment	Estrogen receptor	Growth in SCID mice[a]
7326	Invasive Lobular Ca	–	+	3/4
7356[b]	Invasive Ductal Ca (IDC)	+	+	2/4
7418[b]	IDC	-	-	1/2
7421	Intraductal Ca (with microinvasion)	-	-	1/2
7443[b,c,d]	IDC	+	-	4/4
7486[c,e]	IDC	-	-	5/5
7526	IDC	-	+	2/5
7528	IDC	-	+	3/4
7543	IDC	-	+	3/5
7553	IDC	-	+	2/4
7556[b]	Papillary Cystic Cancer		+	3/5
7573	IDC	-	+	5/8
7582	Infiltrating Lobular Ca	+	+	4/5
7595	IDC	+	-	3/3
7608[b]	IDC	-	+	2/2
7615	IDC	-	+	4/5
7628[c]	IDC	+	-	5/5
7655[f]	IDC			1/3
7661	IDC	+	+	4/8
7679	IDC	+	-	5/9
7717[c]				2/2
7722[c,e]	IDC	N.A.	-	7/7
7744	Intraductal CA (with microinvasion)	-	-	6/10
7748	Infiltrating Lobular Ca	+	+	4/4
7764[c]	IDC	+	-	10/10
7784			+	2/2
7788			+	5/5
7806			+	3/3
7806				0/3
7821			+	1/2
7842			+	3/3
7869				7/8
7882			+	2/2
7892			+	0/3
7897			+	1/3
7914[c]	IDC	+	+	4/5
7929[c]	IDC	-	-	3/5
7960			-	1/2
7967			+	4/5
8029				2/5
8038[c]				4/5
8070				1/2
8099[c]				5/5

[a] Numbers refer to the animals in which palpable growth was obtained in the first passage out of the total number implanted with pieces of the original surgical biopsy material.

[b] Tumors that expressed Type II keratin and were demonstrated to be human by *in situ* hybridization using a human DNA specific probe.

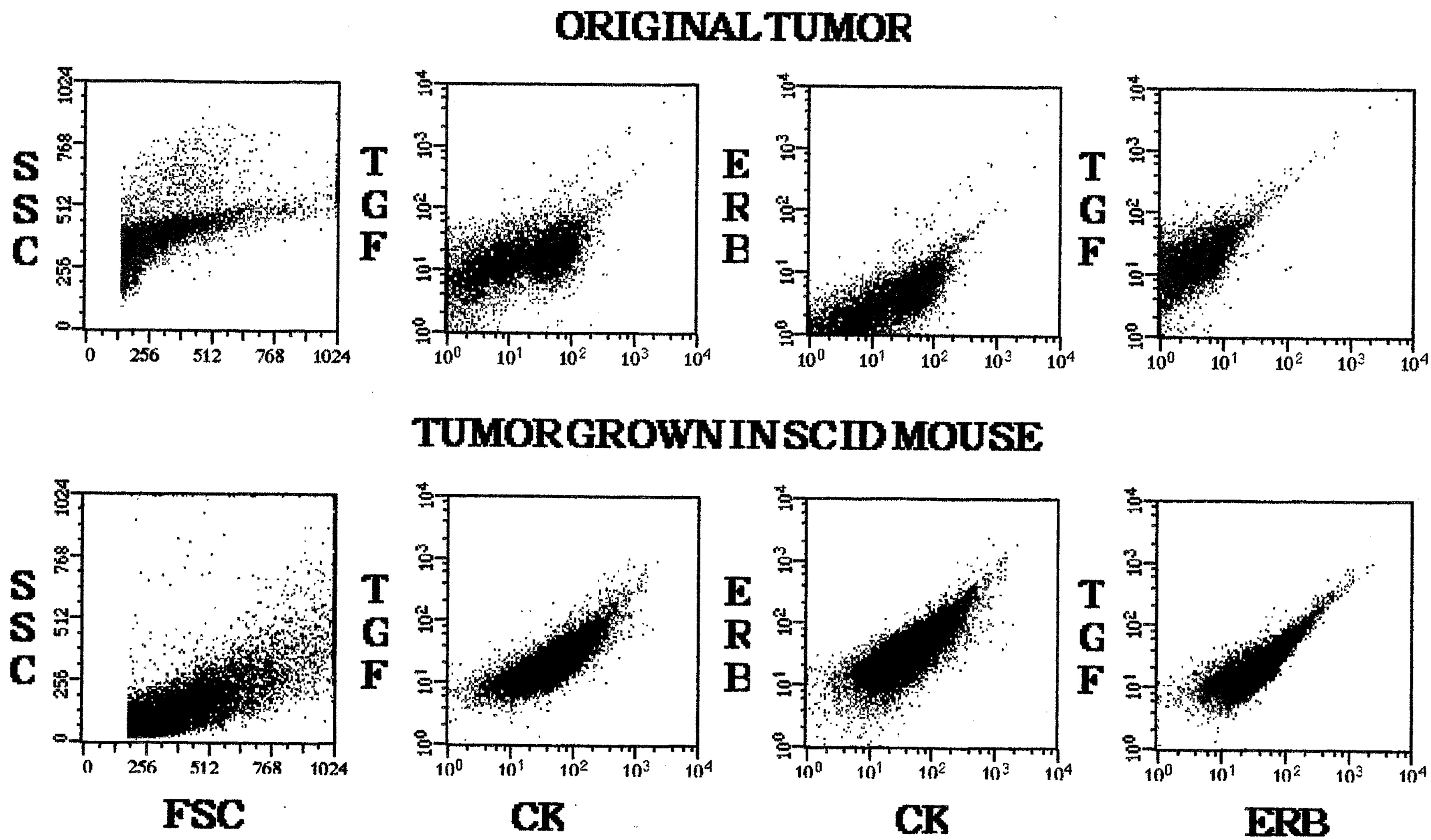
[c] Showed evidence of exceptionally fast growth.

[d] Also grew in nude mice (#7356, #7418 and #7628 did not grow in nude mice).

[e] Showed evidence of metastasis to other organs in later passages.

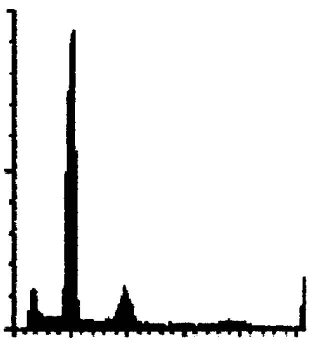
[f] During 2nd passage, grew out as a lymphoma.

DETERMINATION OF CYTOKERATIN, TGF-α, AND c-erb/B2 IN HUMAN BREAST TUMOR



DNA CONTENT OF HUMAN BREAST TUMORS GROWN IN SCID MICE

ORIGINAL TUMOR



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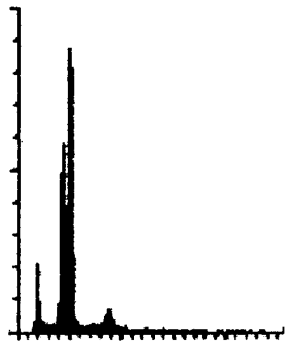

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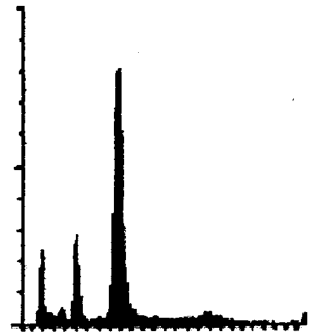

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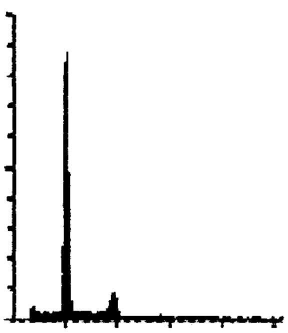
SCID MOUSE UNTREATED



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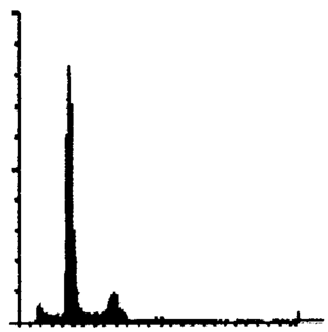

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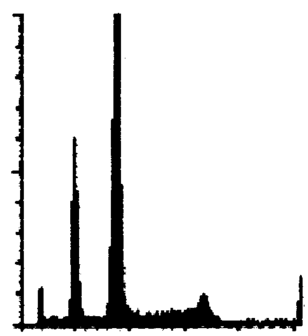

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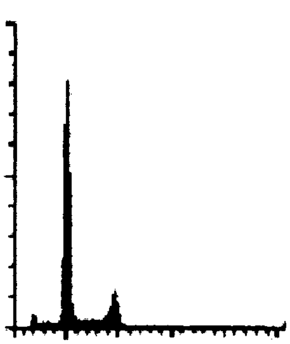
SCID MOUSE ADRIAMYCIN TREATED



DI = 1.00
%S = 14.3

DI = 0.92
%S = 11.0

DI = 1.76
%S = 10.7

421

Liver metastasis and adhesion to the sinusoidal endothelium by human colon cancer cells is related to mucin carbohydrate chain length. Bresalier RS. Byrd JC, Itzkowitz SH, and Brodt P. Henry Ford Health Sciences Ctr, Detroit, Mt. Sinai Med. Ctr. NY and McGill Univ, Montreal.

While patients with mucinous colon cancer have a poor prognosis, it is not known which steps in metastasis depend on specific alterations in mucin glycoproteins. We therefore studied the ability of clonal colon cancer cells which secrete mucins of varying carbohydrate length to adhere to basement membranes and hepatic endothelium, and to metastasize in athymic mice. Cell line LS-C produces mucins containing short carbohydrate chains and expresses high levels of core antigens (sialyl Tn), while LS-B mucin has long carbohydrate chains expressing structures such as sialyl Le^x. LS-B synthesizes 2-4 fold more mucin than LS-C which is inhibited by benzyl-alpha-GalNAc (80% for LS-B vs 23% for LS-C). Adhesion to Matrigel was 2-fold greater for LS-C and this was inhibited in a dose-dependent manner by antibody to sialyl Tn. In contrast, adhesion to an E-selectin-Fc chimera (but not P- or L-selectin) in vitro was 3.5 fold greater for LS-B. This was mimicked by 3-fold greater adhesion to cytokine-stimulated hepatic endothelial cells from athymic mice. LS-B metastasized from cecum to liver of nude mice 4 times more frequently and colonized the liver after splenic-portal injection to a significantly greater extent than LS-C (219 vs 15 hepatic tumor nodules, n=11). Conclusion: Peripheral oligosaccharide structures on the long chain carbohydrates of mucin are important in liver colonization by human colon cancer cells, due in part to their interaction with selectins of the sinusoidal endothelium.

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Stimulation of human ovarian cancer cell growth in vivo with TNFa or IL-1 in immunodeficient scid mice.

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Ovarian cancer tissues express receptors for tumor necrosis factor alpha (TNFa). Both TNFa and IL-1 stimulate proliferation of ovarian cancer cells in vitro. Anti-TNFa antibody or TNFa soluble receptor neutralized TNFa-induced proliferation, as well as proliferation induced by IL-1, suggesting that TNFa may function as an autocrine growth factor for ovarian cancer. We evaluated the effect of recombinant human TNFa and recombinant human IL-1 on the growth of OVCAR-3 ovarian cancer cells in immunodeficient scid mice. Mice were injected intraperitoneally with 30 x 10^6 OVCAR-3 tumor cells. Cytokines were administered intraperitoneally twice per week at a dose of 0.25 or 2.5 mg per mouse for TNFa or 1 mg per mouse for IL-1. The weight of solid peritoneal tumor implants was determined approximately 30 days after tumor cell injection. TNFa or IL-1 significantly stimulated tumor growth ($p<0.05$ and $p<0.02$ respectively, Student's t test). Anti-human TNFa monoclonal antibody (50 mg per mouse) reversed the growth stimulatory effect of TNFa (p=0.02, ANOVA). Consequently, TNFa can stimulate human ovarian cancer growth in vivo as well as in vitro.

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Loss of the tumorigenic phenotype in human bronchial epithelial cancer cell lines passaged *in vitro* but not *in vivo*. Schiller, J.H., Bittner, G., Wu, S.Q., Meisner, L. Department of Human Oncology, University of Wisconsin Comprehensive Cancer Center, Madison, WI 53792.

We have established and characterized an immortalized HBE, nontumorigenic cell line (NL20) which subsequently spontaneously progressed to become tumorigenic (NL20T) (Schiller, JH *et al* Proc AACR 35:163, 1994). These NL20T cells have since been passaged in mice and on plastic tissue culture dishes. Two cell lines have been established from NL20T cells reinoculated in mice (NL20T-A and NL20T-B); these have remained tumorigenic at passage 31. However, the parent NL20 cell line, which has continued to be cultured *in vitro*, has lost its tumorigenicity at late passage. Furthermore, the tumorigenic cell line, NL20T, has also lost its tumorigenicity at passage 20 (NL20T-n) when cultured *in vitro*. Karyotypic analysis revealed that early passage, tumorigenic NL20T cells, when compared to nontumorigenic NL20 cells, acquired 6 new markers which resulted in loss of segments of chromosomes 13 and 18, and amplification of 9q21.2→q34. Cytogenetic differences between the revertant nontumorigenic NL20T-n cells and the tumorigenic NL20T-A and B cells include maintenance of the amplified 9q21.2→q34 and an extra chromosome 19 in the tumorigenic cells. Comparison of these well characterized tumorigenic and nontumorigenic parent and revertant cell lines, all derived from the same parent cell, will allow us to determine specific genetic and cellular events associated with acquisition and loss of the tumorigenic phenotype.

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Characterization of a neuroblastoma xenograft model. Vassal, G., Terrier-Lacombe, M.J., Vénuat, A.M., Bénard, J., Morizet, J., Ardouin, P., Comoy, E., Valteau, D., Gouyette, A. Institut Gustave Roussy, Villejuif; France

In order to establish neuroblastoma (NB) xenografts for preclinical phase II studies, 14 primary NB tumors (5 stage II, 6 stage III, 3 stage IV) and 1 IVs subcutaneous metastasis were transplanted subcutaneously in irradiated female athymic Swiss mice. Thirteen tumors were obtained at diagnosis, 1 after chemotherapy and 1 at relapse. Two out of 15 NBs showed N-myc amplification. Three xenografts were obtained: IGRNB3, 8 and 15. Human origin was confirmed by cytogenetic and LDH isozymes analysis. IGRNB3 and IGRNB8 derived from newly diagnosed stage III NBs with N-*myc* amplification. IGRNB3 was a diploid NB with 1p deletion, double minutes and N-*myc* amplification (14 copies). IGRNB8 was a paradiploid NB with 1p deletion and N-*myc* amplification (5 copies). High levels of catecholamines were found in urine of tumor-bearing mice. Median tumor doubling times were 11 and 3 days for IGRNB3 and IGRNB8, respectively. *In vivo* passages were successful beyond #7. The most recent xenograft, IGRNB15, derived from a newly diagnosed diploid stage II NB with 1p deletion and no N-*myc* amplification in a 4 month-old boy. These xenografts are currently used for the evaluation of DNA-topoisomerase I inhibitors.

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Cloning of the TIMP-1 Genomic Regulatory-Promoter from PC-3 Human Prostate Tumor Cells.

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We have recently cloned the human genomic TIMP-1 gene (4 Kb) including the coding and regulatory-promoter regions from highly non-invasive PC-3 human prostate sublines (see Wang and Stearns, 1991, Differentiation, 48: 115). We have characterized the regulatory region by restriction endonuclease mapping and identification of potential regulatory sequences based on gel retardation, DNA footprinting, and pCAT transfection assays. A 18 bp cis-acting regulatory element (ATGCCACGATGACTCATCA) has been identified 748 bp upstream of the 5' ATG start site which strongly upregulates pCAT activity in transfected PC-3 ML clones in response to IL-10 (10- 20 ng/ml-2 hr) and IL-6. Currently we are subcloning regions of the 5' promoter region for sequencing, in vitro transcription and in vivo transfection analysis. These experiments should demonstrate if independent regulatory sequences (and factors) control TIMP-1 synthesis. Supported by NIH-NCI grant CA 57180 to MES.

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Coengraftment of human primary breast carcinoma and autologous immunocompetent cells in severe combined immunodeficient mice. Xu Y., Raza-Egilmez S., Sakakibara T., Bankert R.B., and Repasky E.A.; Dept. of Molecular Immunology, Roswell Park Cancer Institute, Buffalo, NY 14263.

We have recently developed a protocol for the growth and passage of human primary breast carcinoma by embedding small pieces of surgical specimen into the gonadal fat pad (GFP) of severe combined immunodeficient (SCID) mice. Using this protocol, we have observed that the histology of the various tumor xenografts and their extent of differentiation can differ considerably, suggesting that breast tumor growth within SCID mice may recapitulate biological properties of the original tumors. We now report the presence of large numbers of lymphocytes and plasma cells that are among the tumor cells; these cells can be visualized for several months following the original engraftment and also in subsequent passages. Immunofluorescent markers indicate that these lymphoid cells are mostly human in origin. ELISA analysis of the sera from engrafted mice shows that the majority of the sera contained human immunoglobulin at range of 1.0 to 300 μg / ml that is probably expressed by the human plasma cells seen within the tumor. Western blotting analysis shows that human breast tumor antigens can be recognized by these antibodies. We believe that this new SCID-human chimera model will prove valuable in the exploration of the pathogenesis of human breast cancer, in the understanding of immunological responses to the malignant cells, and in the testing of new therapeutic approaches for treatment of this disease. Supported by grants from the U.S. Army Medical Research & Development Command, and National Live Stock & Meat Board.